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Reverse Transcription Activity from *Bacillus Stearothermophilus* DNA Polymerase in the Presence of Magnesium

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to US Serial No. 60/135,437 filed May 22, 1999.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

BACKGROUND OF THE INVENTION

The present invention is in the fields of molecular and cellular biology. The invention is generally related to a thermostable reverse transcriptase derived from *Bacillus stearothermophilus* and methods for the reverse transcription of nucleic acid molecules. Specifically, the invention relates to methods for producing nucleic acid molecules (particularly cDNA molecules) using a thermostable protein fragment having reverse transcriptase activity in the presence of magnesium ions and in the substantial absence of manganese ions. The invention also provides methods for the amplification of a DNA segment from an RNA template using combinations of reverse transcriptase and thermostable DNA polymerase enzymes using a thermostable reverse transcriptase derived from *Bacillus stearothermophilus*.

Reverse Transcription of RNA

The term "reverse transcriptase" describes a class of polymerases characterized as RNA-dependent DNA polymerases. All known reverse transcriptases require a primer to synthesize

a DNA transcript from an RNA template. Reverse transcriptase has been used primarily to transcribe RNA into cDNA, which can then be cloned into a vector for further manipulation or used in various amplification methods such as RT-PCR, NASBA, TMA, 3SR, or SPSR.

Reverse transcription is commonly performed with viral reverse transcriptases isolated from Avian myeloblastosis virus (AMV-RT) or Moloney murine leukemia virus (MMLV-RT), which are active in the presence of magnesium ions.

Reverse transcription at higher temperatures is advantageous to overcome secondary structures of the RNA template which could result in premature termination of products. For this reason, a reverse transcription reaction may begin with an RNA denaturation step which can be carried out, for example, by heating to a temperature generally at least 60°C. Unfortunately, both the AMV-RT and MMLV-RT (RNase H^+ or RNase H^- forms) are inactivated at elevated temperatures, each having a temperature optimum between 48-55°C or 37-42°C, respectively.

Alternative methods are described in U.S. Pat. Nos. 5,310,652 and 5,322,770 using the reverse transcriptase activity of DNA polymerases of thermophilic organisms which are active at higher temperatures, both of which are incorporated herein by reference. Thermostable DNA polymerases with reverse transcriptase activities are commonly isolated from Thermus species. These DNA polymerases, however, possess significant reverse transcriptase activity only in the presence of manganese ions. These reaction conditions are suboptimal because, in the presence of manganese ions, the polymerase copies the template RNA with low fidelity and the RNA template is prone to increased degradation (Beckman R.A., et al., *Biochem* 24:5810-5817 (1985); Ricchetti M. and Buc H., *EMBO J.* 12:387-396 (1993)).

PCR Amplification of RNA

Reverse transcriptases have been extensively used in reverse transcribing RNA prior to PCR amplification. This method, often referred to as RT-PCR, is widely used for detection and quantitation of RNA. In RT-PCR, an RNA template is first copied into cDNA using a reverse transcriptase, a reaction termed "first-strand synthesis." PCR is then performed to exponentially amplify the cDNA (*see* U.S. Pat Nos. 4,683,195 and 4,683,202).

In its least sophisticated implementation, the RT-PCR method entails three steps, namely: (1) denaturation of the RNA by heating; (2) synthesis of the first cDNA strand ("first-strand synthesis") in a buffer containing, apart from the nucleoside triphosphates, a first primer capable of hybridizing with a sequence located in the vicinity of the 3' end of the RNA template, and a reverse transcriptase; and (3) synthesis of the second cDNA strand ("second-strand synthesis") by addition of a second primer capable of hybridizing with a sequence adjoining the 3' end of the first cDNA strand (i.e., the primer must be identical or sufficiently homologous to a sequence adjoining the 5' end of the RNA template) and a DNA polymerase, followed by the succession of PCR amplifications (Schwartz, S. *J. Virol.*, 24(6):2519-2529 (1990)).

To attempt to address the technical problems often associated with RT-PCR, a number of protocols have been developed, where the above three step procedure has been reduced to a "two-step" or a "one-step" protocol.

In the so-called "uncoupled" RT-PCR method ("two-step"), the first-strand synthesis (cDNA) reaction is performed in one tube. Following cDNA synthesis, the reaction is diluted into PCR reaction mixtures to decrease $MgCl_2$ and deoxyribonucleoside triphosphate (dNTP) concentrations to conditions optimal for *Taq* DNA polymerase activity, and aliquots are then

diluted in separate tubes. By contrast, "coupled" RT-PCR methods ("two-step") use a common or compromised buffer for reverse transcriptase and *Taq* DNA polymerase activities. In one version, after the first-strand synthesis reaction, the tubes are opened and the DNA polymerase(s) and other PCR reagents are added (Goblet, C. et al., *Nucl. Acids Res.* 17(5):2144 (1989)). In another version, the reverse transcriptase activity is a component of the thermostable *Tth* DNA polymerase. Annealing and cDNA synthesis are performed in the presence of manganese ions, then PCR is carried out in the presence of magnesium after the removal of manganese by a chelating agent (Myers, T.W., et al., *Biochem.* 30:7661-7666 (1991)). Finally, the "continuous" RT-PCR method ("one-step") integrates the three RT-PCR steps into a single continuous reaction that avoids the opening of the reaction tube. Continuous RT-PCR has been described as a single enzyme system using the reverse transcriptase activity of the thermostable DNA polymerase *Tth* and as a two-enzyme system using AMV-RT and *Taq* DNA polymerase wherein the initial 65°C RNA denaturation step was omitted.

Each of the above protocols has significant disadvantages. Manganese-dependent reverse transcription and subsequent amplification with a thermostable DNA polymerase such as *Tth* results in a significantly increased risk of degradation. Moreover, in the presence of manganese ions, the polymerase copies the template RNA with low fidelity. The use of viral reverse transcriptases such as AMV-RT and MMLV-RT, which are not dependent upon manganese for reverse transcriptase activity, are inactivated at the higher temperatures necessary to overcome secondary structures of RNA templates which could result in premature termination of products.

Transcription-based Amplification of RNA

Reverse transcriptases also have been used to reverse transcribe RNA during transcription-based amplification techniques, where these techniques may be classified either as temperature cycling reactions or as isothermal reactions. Isothermal amplifications are conducted at essentially constant temperature, in contrast to the cycling between high and low temperatures characteristic of amplification reactions such as the PCR.

An example of a transcription-based amplification technique using temperature cycling is the transcription-based amplification system (TAS), which is described in U.S. Pat. No. 5,437,990 and incorporated herein by reference, and consists of the repetition of a cycle with three stages. The first stage makes it possible to synthesize a cDNA from RNA in the presence of reverse transcriptase and a hybrid deoxynucleotide primer containing a specific sequence of phage RNA polymerase promoter. Following the thermal denaturation of the RNA/cDNA heteroduplex, the single-stranded cDNA is replicated by reverse transcriptase in the presence of an anti-sense oligonucleotide primer. The DNA homoduplex thus obtained during this second stage contains a double-stranded promoter to which a phage DNA-dependent RNA polymerase can bind. The third stage then consists of transcribing RNA molecules (from 30 to 1000 per template) which will again be able to serve as template for the synthesis of cDNA and thereby to continue the amplification cycle (Kwoh, D.Y., et al., *Proc. Natl. Acad Sci. USA* 86:1173-1177 (1989)).

In contrast, various methods have been derived from TAS that are isothermal amplifications such as Nucleic Acid Sequence-Based Amplification (NASBA) which is described in U.S. Pat. Nos. 5,130,238 and 5,409,818, both of which are incorporated herein by reference, Transcription Mediated Amplification (TMA) which is described in U.S. Pat. No. 5,399,491 and incorporated herein by reference, Self-Sustained Sequence Replication (3SR)

discussed by Guatelli, J.C., et al. in *Proc. Natl. Acad. Sci USA* 87, 1874-1878 (1990), with an erratum at *Proc. Natl. Acad. Sci.USA*, 87:7797 (1990), which is incorporated herein by reference, and Single Primer Sequence Replication (SPSR) which is described in U.S. Pat. No. 5,194,370 and incorporated herein by reference.

These methods have in common the combination of three enzymatic activities: RNA- and DNA-dependent DNA polymerase (a retrovirus reverse transcriptase such as AMV-RT or MMLV-RT), ribonuclease H (RNase H) (*E. coli* enzyme and/or enzymatic activity associated with reverse transcriptase), and DNA-dependent RNA polymerase (*e.g.*, T7 bacteriophage RNA polymerase). These methods are based on the same principle and are carried out at a fixed temperature (from 37°C to 45°C), according to a continuous process of reverse transcription and transcription reactions in order to replicate an RNA target via cDNA. As in the case of TAS, an RNA polymerase (*e.g.*, T7 phage) binding site is introduced into the cDNA by the primer used for the reverse transcription stage. However, the denaturation of the RNA/cDNA heteroduplex is carried out isothermally by specific hydrolysis of the RNA of this heteroduplex by RNase H activity. The free cDNA is then replicated from a second oligonucleotide primer by reverse transcriptase. The DNA/DNA homoduplex is transcribed into RNA by T7 RNA polymerase and this RNA can again serve as template for the next cycle.

While the NASBA, TMA, 3SR, and SPSR systems are all able to generate a large quantity of product, one or more of the enzymes involved in each cannot be used at high temperatures (*i.e.*, >45°C). Therefore, the reaction temperatures cannot be raised to prevent, for example, non-specific hybridization of the primers. If the primers are shortened in order to make them melt more easily at low temperatures, the likelihood of having more than one perfect match in a complex genome increases. Finally, reactions not carried out at high temperatures

ineffectively denature, if at all, RNA secondary structure.

A thermostable reverse transcriptase that is active in the presence of magnesium ions and in the substantial absence of manganese ions would overcome the disadvantages associated with low temperature nucleic acid amplification reactions, and as such, would greatly enhance amplification methods such as RT-PCR, NASBA, TMA, 3SR, or SPSR. Among the advantages of such a reverse transcriptase are: (1) improved ability to reverse transcribe RNA molecules with greater secondary structure, especially due to the use of higher reaction temperatures; (2) greater stability of the reverse transcriptase during performance of reactions at elevated temperatures; (3) longer shelf-life of the reverse transcriptase due to greater thermostability; (4) greater accuracy of the reverse transcription product (cDNA) due to the higher fidelity of the reverse transcriptase; and/or (5) synthesis of larger amounts of cDNA due, in part, to lesser amounts of RNA substrate degradation.

BRIEF SUMMARY OF THE INVENTION

The present invention is generally directed to thermostable DNA polymerases from *Bacillus stearothermophilus* (*Bst*) which are mutated or truncated forms of the native enzyme containing a deletion in the 5'-3' exonuclease domain of the enzyme and/or its corresponding gene, and which exhibit reverse transcriptase activity, preferably in the presence of magnesium ions and in the substantial absence of manganese ions. These enzymes may be used in first strand cDNA synthesis and other biochemical protocols that require a reverse transcriptase activity. Furthermore, because the enzymes provided herein are thermostable, they are suitable for use in biochemical applications using higher temperatures than many other reverse

transcriptases, such as AMV-RT and MMLV-RT.

In a preferred aspect of the invention, a mutated or truncated form of the native *Bst* DNA polymerase has a molecular mass of about 55 to 65 kDA as determined by SDS gel electrophoresis.

In another preferred aspect of the invention, the concentration of magnesium-containing molecules is at least 1 mM, and more preferably about 1.0 mM to about 10.0 mM, about 1.0 mM to about 5 mM, and most preferably about 1.0 mM to about 2.0 mM, or about 1.5 mM. The invention also is directed to such concentrations wherein the source of the magnesium-containing molecules is a buffer or a magnesium-containing salt which may be magnesium chloride, magnesium sulfate, or magnesium acetate, as well as other magnesium-containing buffers and salts that will be familiar to one of ordinary skill.

Additionally, the invention is directed to methods for amplifying a nucleic acid molecule comprising (a) mixing an RNA template with a composition comprising a truncated form of *Bst* DNA polymerase (sold as ISOTHERM™ DNA polymerase, Epicentre Technologies) having reverse transcriptase activity in the presence of magnesium ions and in the substantial absence of manganese ions in combination with one or more DNA polymerases to form a mixture; and (b) incubating the mixture under conditions sufficient to amplify a DNA molecule complementary to all or a portion of the RNA template. In preferred methods, the DNA polymerases used are thermostable DNA polymerases, and most preferably *Tne*, *Tma*, *Taq*, *Pfu*, *Tth*, *Pwo*, *Tfl*, or a mutant, variant or derivative thereof.

In other preferred aspects of the invention, the DNA polymerases may comprise a first DNA polymerase having 3' exonuclease activity, most preferably a DNA polymerase selected from the group consisting of *Pfu*, *Pwo*, *Tne*, *Tma*, and mutants variants and derivatives thereof,

and a second DNA polymerase having substantially reduced 3' exonuclease activity, most preferably a DNA polymerase selected from the group consisting of *Taq*, *Tfl*, *Tth*, and mutants, variants and derivatives thereof. In additional preferred aspects of the invention, the unit ratio of the reverse transcriptase to the DNA polymerases is from about 0.25:1 to about 16:1, and most preferably a ratio of about 4:1.

The invention also is directed to such methods wherein the mixture further comprises one or more nucleotides, preferably deoxyribonucleoside triphosphates (most preferably dATP, dUTP, dTTP, dGTP or dCTP), dideoxyribonucleoside triphosphates (most preferably ddATP, ddUTP, ddGTP, ddTTP, or ddCTP) or derivatives thereof. Such nucleotides may optionally be detectably labeled (*e.g.*, with a radioactive or non-radioactive detectable label).

The invention also is directed to such methods wherein such mixture further comprises one or more oligonucleotide primers, which are preferably oligo(dT) primers, random primers, arbitrary primers or target-specific primers, and which is more preferably a gene-specific primer.

The invention also is directed to such methods wherein the incubating step comprises (a) incubating the mixture at a temperature of at least 40°C, most preferably with a range of at least about 40°C to about 80°C, and for a time sufficient to make a DNA molecule complementary to all or a portion of the RNA template; and (b) incubating the DNA molecule complementary to the RNA template at a temperature and for a time sufficient to amplify the DNA molecule, preferably via thermocycling, more preferably thermocycling comprising alternating heating and cooling of the mixture sufficient to amplify said DNA molecule, and most preferably thermocycling comprising alternating from a first temperature range of from about 90°C to about 100°C, to a second temperature range of from about 45°C to about 75°C, preferably from about 60°C to about 75°C. In particularly preferred aspects of the invention, the thermocycling is

performed greater than 20 times, more preferably greater than 30 times.

In a further aspect, the present invention is directed to methods for amplifying a nucleic acid molecule using transcription-based amplification techniques that include, but are not limited to, NASBA, TMA, 3SR, or SPSR.

A part of the invention includes test kits for carrying out the previously described methods.

Other preferred embodiments of the present invention will be apparent to one of ordinary skill in light of the following drawings and description of the invention, and of the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a photograph of an ethidium bromide stained gel demonstrating the ability of both full-length *Bst* DNA polymerase and the *Bst* large fragment to produce a 1375 bp cDNA copy of the 16S ribosomal RNA of *E. coli*. Lane M contains a DNA sizing ladder. Lanes 1 and 2 contain the cDNA produced by full-length *Bst* DNA polymerase. Lanes 3 and 4 contain the cDNA produced by the *Bst* large fragment.

Figure 2 is a photograph of an ethidium bromide stained gel demonstrating the effect of magnesium concentration on cDNA synthesis. Reverse transcription of the 16S ribosomal RNA of *E. coli* with the large fragment of *Bst* DNA polymerase, in the presence of magnesium, results in a 1375 b cDNA. Lane M contains a 100 bp DNA sizing ladder; Lanes 1-4 contain 0, 1.0, 2.0, and 3.0 mM $MgCl_2$ respectively.

Figure 3 is a photograph of an ethidium bromide stained gel demonstrating coupled reverse transcription, in the presence of magnesium or manganese, using the large fragment of *Bst* DNA polymerase, and *Taq* DNA polymerase for the PCR amplification. The 463 bp

amplification product of a region of the tobacco mosaic virus (TMV) RNA is indicated with an arrow. Lane M contains 100 bp DNA sizing ladder; Lane 1 contains the reaction with no magnesium or manganese present during reverse transcription; in Lane 2 the RT reaction contained only 1.5 mM $MgCl_2$; in Lane 3 the RT reaction contained only 0.5 mM $MnSO_4$.

Figure 4 is a photograph of an ethidium bromide stained gel demonstrating a long RNA amplification with the large fragment of *Bst* DNA polymerase in the presence of magnesium, coupled with a mix of thermostable DNA polymerases. Continuous reverse transcription and PCR amplification of a region of TMV RNA with the large fragment of Bst DNA polymerase, *Taq* DNA polymerase and *Pwo* DNA polymerase, results in a 4650 bp product. Lane M contains a DNA sizing ladder; Lanes 1 and 2 are the products from reactions containing 0X and 1X MASTERAMP PCR Enhancer (Epicentre Technologies) respectively.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to a thermostable and enzymatically active truncated fragment derived from native full-length *Bacillus stearothermophilus* DNA polymerase for use in reverse transcription and/or reverse transcriptase-polymerase chain reaction (RT-PCR), where it has been unexpectedly discovered that said fragment has significant reverse transcriptase activity in the presence of magnesium ions and in the substantial absence of manganese ions. The invention also provides compositions comprising *Bst* enzymes having reverse transcriptase activity, one or more DNA polymerases, one or more primers, one or more nucleotides, and a suitable buffer. These compositions may be used in the methods of the invention to produce,

analyze, quantitate and otherwise manipulate nucleic acid molecules using a one- or two-step RT-PCR procedure.

Reverse Transcriptase Enzymes

The present invention relates to a thermostable and enzymatically active truncated fragment derived from native full-length *Bacillus stearothermophilus* DNA polymerase ("*Bst* large fragment") having reverse transcriptase activity in the presence of magnesium ions and in the substantial absence of manganese ions. *Bst* DNA polymerase type strain 5, ATCC number 12980, was obtained from the American Type Culture Collection, Rockville, Md. Using procedures well known in the art, a genomic library was prepared from said strain that led to the identification of a clone that expressed the full-length *Bst* DNA polymerase. The full-length protein may be purified according to any number of protocols known in the art (*see e.g.*, Ye, S.Y. and Hong, G.F., *Scientia Sinica*, 30:503 (1987) and U.S. Pat. No. 5,874,282, col. 11, lines 3-51).

The purified full-length *Bst* DNA polymerase (167 μg/ml) (microgram/ml) in a standard storage buffer (50% (v/v) glycerol solution; 0.05 M Tris-HCl pH 7.5; 0.1 mM EDTA; 1 mM DTT; 0.1 M NaCl; and 0.1% Triton X-100) was treated with 0.01 volumes of 25 μg/ml (microgram/milliliter) subtilisin for 16 hours at room temperature. The proteolysis reaction was terminated by adding 0.01 volumes of 100 mM PMSF in absolute ethanol followed by mixing. The solution was diluted with 1 volume of water and subjected to chromatography on a Bio-Rex 70 Ion Exchange Column (Bio-Rad Laboratories, Hercules, CA). The enzyme which bound to the column under these conditions was subsequently eluted with a linear NaCl gradient for 0.05 to 0.5 M in a chromatography buffer (0.05 M Tris-HCl pH 7.5; 0.1 mM EDTA; 1% (v/v) β-mercaptoethanol; and 5% (v/v) glycerol). The fractions were assayed for *Bst* polymerase activity

and for purity by 10% SDS electrophoresis before pooling. The pool was dialyzed against the above described standard storage buffer.

Subsequent analysis of the active, truncated large fragment revealed (1) that the fragment has a molecular mass of about 55 to 65 kDA as determined by 10% SDS PAGE, (2) that the fragment lacks 5'-3' exonuclease activity, and (3) that the fragment has reverse transcriptase activity in the presence of magnesium ions and in the substantial absence of manganese ions.

Particularly preferred enzymes for use in the invention include *Bst* reverse transcriptases, but are not necessarily limited to, commercially available enzymes such as IsoTherm™ DNA polymerase, available from Epicentre Technologies Corp., Madison, WI.

Compositions

The buffer in the compositions of the invention provide appropriate pH and ionic conditions for *Bst* enzymes having reverse transcriptase activity and DNA polymerase enzymes. The nucleotides used in the compositions (e.g., deoxyribonucleoside triphosphates (dNTPs)), and the primer nucleic acid molecules provide the substrates for synthesis or amplification of nucleic acid molecules in accordance with the invention.

Buffer and Ionic Conditions

The buffer and ionic conditions of the present compositions have been optimized to yield total and full-length cDNA product in reverse transcription and amplification reactions. Preferred compositions of the invention provide a concentration of magnesium-containing molecules of at least 1 mM, and more preferably about 1.0 mM to about 10.0 mM, about 1.0 mM to about 5 mM, and most preferably about 1.0 mM to about 2.0 mM, or about 1.5 mM. The invention also is directed to such concentrations wherein the source of the magnesium-containing

molecules is a buffer or a magnesium-containing salt which may be magnesium chloride, magnesium sulfate, or magnesium acetate, as well as other magnesium-containing buffers and salts that will be familiar to one of ordinary skill.

DNA Polymerases

The compositions of the invention also comprise one or more DNA polymerases, which are preferably thermostable DNA polymerases. These DNA polymerases may be isolated from natural or recombinant sources, by techniques well-known in the art, from a variety of thermophilic bacteria that are available commercially, or may be obtained by recombinant DNA techniques. Suitable for use as sources of thermostable polymerases or the genes thereof for expression in recombinant systems are the thermophilic bacteria *Thermus aquaticus, Thermus thermophilus*, *Thermococcus litoralis*, *Pyrococcus furiosus*, *Pyrococcus wosei* and other species of the *Pyrococcus* genus, *Bacillus stearothermophilus*, *Sulfolobus acidocaldarius*, *Thermoplasma acidophilum*, *Thermus flavus*, *Thermus ruber*, *Thermus brockianus*, *Thermotoga neapolitana*, *Thermotoga maritima* and other species of the *Thermotoga* genus, and *Methanobacterium thermoautorophicum*, and mutants variants or derivatives thereof. It is to be understood, however, that thermostable DNA polymerases from other organisms may also be used in the present invention without departing from the scope or preferred embodiments thereof.

Thermostable DNA polymerases such as *Taq* is preferably added to the present compositions at a final concentration in solution of about 25-100 units per milliliter, most preferably 100 units per milliliter, and *Bst* large fragment (sold as ISOTHERM DNA polymerase, Epicentre Technologies) is preferably added to the present compositions at final concentration in solution of about 25-400 units per milliliter, most preferably 400 units per milliliter.

In preferred compositions of the invention, the concentration of DNA polymerases is

determined as a ratio of the concentration of the enzymes having reverse transcriptase activity. Thus, in particularly preferred compositions the ratio units of the *Bst* large fragment having reverse transcriptase activity to *Taq* DNA polymerase ranges from about 0.25:1 to about 16:1, most preferably a ratio of about 4:1. Of course, other suitable ratios of unit activities of reverse transcriptase enzymes to DNA polymerases suitable for use in the invention will be apparent to one of ordinary skill in the art.

dNTPs

The compositions of the invention further comprise one or more nucleotides (e.g., deoxyribonucleoside triphosphates (dNTPs)). The nucleotide components of the present compositions serve as the building blocks for newly synthesized nucleic acids, being incorporated therein by the action of the reverse transcriptases or DNA polymerases. Examples of nucleotides suitable for use in the present compositions include, but are not limited to, dUTP, dATP, dTTP, dCTP, dGTP, dITP, 7-deaza-dGTP, α-thio-dATP, α-thio-dTTP, α-thio-dGTP, α-thio-dCTP or derivatives thereof, all of which are available commercially from various suppliers. The dNTPs may be unlabeled, or they may be detectably labeled by coupling them by methods known in the art with radioisotopes, vitamins, fluorescent moieties, chemiluminescent labels, dioxigenin and the like. Labeled dNTPs may also be obtained from commercial suppliers. In the compositions, the dNTPs are added to give a working concentration of each dNTP of about 200 μM (micromolar). Other suitable working concentrations will be apparent to one of ordinary skill in the art.

Primers

In addition to nucleotides, the present compositions comprise one or more primers which facilitate the synthesis of a first strand DNA molecule complementary (single-stranded cDNA

molecule) to all or a portion of an RNA template. Such primers may also be used to synthesize a DNA molecule complementary to all or a portion of the first strand DNA molecule, thereby forming a double-stranded cDNA molecule. Additionally, these primers may be used in amplifying nucleic acid molecules in accordance with the invention. Such primers include, but are not limited to, target-specific primers (which are preferably gene-specific primers), oligo(dT) primers, random primers or arbitrary primers. Additional primers that may be used for amplification of the DNA molecules according to the methods of the invention will be apparent to one of ordinary skill in the art.

Methods of RT-PCR

In the RT-PCR reaction, the reaction mixtures are incubated at a temperature sufficient to synthesize a DNA molecule complementary to all or a portion of the RNA template. Such conditions typically occur at temperatures of at least 40°C, and more preferably range from at least about 40°C to about 80°C. After the reverse transcription reaction, the reaction is incubated at a temperature sufficient to amplify the synthesized DNA molecule. Preferably the amplification is accomplished via one or more polymerase chain reactions (PCRs). Preferred conditions for amplification comprise thermocycling, which may comprise alternating heating and cooling of the mixture sufficient to amplify the DNA molecule and which most preferably comprises alternating from a first temperature range of from about 90°C to about 100°C, to a second temperature range of from about 45°C to about 75°C, preferably from about 60°C to about 75°C. According to the invention, the thermocycling may be performed any number of times, preferably from about 5 to about 80 times, more preferably greater than about 20 times and most preferably greater than about 30 times.

The compositions and methods of the present invention may also be used for the production, analysis and quantitation of large nucleic acid molecules (e.g., "long-PCR" or "long RT-PCR"), preferably nucleic acid molecules that are larger than about 3-6 kilobases in size, more preferably larger than about 4-5 kilobases in size, and most preferably nucleic acid molecules that are larger than about 4 kilobases in size. In this aspect of the invention, combinations of DNA polymerases, preferably mixtures of one or more DNA polymerases lacking 3'-5' exonuclease activity ("3'-exo–") with one or more DNA polymerases having 3'-5' activity ("3'-exo+") may be added to the compositions of the invention (Barnes, W.M., *Proc. Natl. Acad. Sci.* 91:2216-2220 (1994)). Preferred 3'-exo- and 3'-exo+ polymerases for use in this aspect of the invention are thermostable polymerases. Particularly preferred 3'-exo– polymerases include, but are not limited to, *Taq*, *Tne* (3'-exo–), *Tma* (3'-exo–), *Pfu* (3'-exo–), and *Pwo* (3'-exo–), or mutants, variants or derivatives thereof. Particularly preferred 3'-exo+ polymerases include, but are not limited to, *Pfu*, *Pwo*, *Tne*, *and Tma*.

Methods of NASBA, TMA, 3SR, or SPSR Amplification:

It will be apparent that, in addition to RT-PCR, the methods of the invention may be easily adapted to other amplification techniques such as NASBA, TMA, 3SR, or SPSR. In a reaction scheme similar to that previously described, the three enzymatic steps, which are carried out at a fixed temperature (from about 37°C to about 45°C), can be modified to function at a higher fixed temperature (from about 40°C to about 80°C). For example, replacing a retrovirus reverse transcriptase (*i.e.*, AMV-RT or MMLV-RT) with a thermostable reverse transcriptase derived from *Bacillus stearothermophilus* also would amplify a DNA molecule complementary to all or a portion of the RNA template. Additionally, a thermostable RNase H as described in

U.S. Pat. Nos. 5,268,289 and 5,459,055 and 5,500,370, all of which are incorporated herein by reference (sold as HYBRIDASE Thermostable RNase H, Epicentre Technologies), can be substituted for a non-thermostable RNase H enzyme derived from *E. coli* or associated with retrovirus reverse transcriptases. Finally, a non-thermostable DNA-dependent RNA polymerase (*e.g.*, T7 bacteriophage RNA polymerase) may be replaced by either (1) a mutant phage RNA polymerase (*e.g.*, mutated forms from T3, T7, or SP6 RNA polymerases) that is active under thermostable conditions (*i.e.*, from about 40°C to about 80°C), wherein each mutant RNA polymerase employs its own specific promoters; or (2) a thermophilic phage RNA polymerase, from native or recombinant sources, that itself encodes for a thermostable RNA polymerase; or (3) a bacterial RNA polymerase from any thermophilic organism (*e.g.*, *Tth* or *Bst*) using promoters from the respective thermophilic organism.

It will be readily apparent to one of ordinary skill in the relevant arts that other suitable modifications and adaptions to the methods and applications described herein are obvious and may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting the invention.

Example 1: Detection of reverse transcriptase activity in presence of magnesium ions

To demonstrate that the large fragment of *Bst* DNA polymerase is capable of magnesium-dependent reverse transcription, first strand synthesis reactions were set-up using 5 units of the large fragment of *Bst* DNA polymerase or the full-length *Bst* protein. A DNA primer, designed to transcribe a region of the 16S rRNA of *E.coli*, was used in a buffered reaction in the presence

of 1.5 mM $MgCl_2$.

Initially, duplicate reactions were performed in RT-PCR buffer containing 50 mM Tris-HCl, (pH 9.0), 20 mM $(NH_4)_2SO_4$, 12.5 mM NaCl. Reactions also contained 200 μM (micromolar) each dNTP, 50 pmoles of 16S rRNA reverse primer (5' AGGCCCGGGAACGTATTCAC 3') (SEQ ID NO: 1), 1 μg (microgram) total *E.coli* RNA, and 5 units of enzyme. The reagents were incubated for 30 minutes at 60°C to allow reverse transcription. Ten microliters of each reaction were then separated by agarose gel electrophoresis and visualized by ethidium bromide staining and UV transillumination. Both Bst DNA polymerase and the *Bst* large fragment produced a cDNA transcript of the appropriate length (1375 nucleotides) as shown in Figure 1, lanes 1-2, and lanes 3-4, respectively.

The optimal magnesium concentration for RT with the *Bst* large fragment was defined with reactions containing varied amounts of $MgCl_2$. Reverse transcription was performed in buffer containing 50 mM Tris-HCl (pH 8.3), and 50 mM KCl. Reactions also contained 200 μM (micromolar) each dNTP, 50 pmoles of 16S rRNA reverse primer (as defined above), 1 μg (microgram) of *E.coli* RNA, and 20 U of *Bst* large fragment. The concentration of $MgCl_2$ added was 0, 1, 2, and 3 mM. Reactions were incubated for 30 minutes at 60°C, and products were separated and visualized by agarose gel electrophoresis. The results are depicted in Figure 2. The optimum concentration of $MgCl_2$ for reverse transcription was 2 mM for reverse transcription with this primer and template pair.

Example 2: RT-PCR using *Bst* reverse transcriptase in the presence of magnesium

Reverse transcription was also demonstrated with another primer and RNA template combination. The reverse transcription and subsequent amplification of a 463 bp region of the

tobacco mosaic virus (TMV) was performed using the magnesium-dependent RT activity of the *Bst* large fragment. Reverse transcription was performed in reactions containing 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 μM (micromolar) each dNTP, 50 pmoles of TMV reverse primer (5' CCCTTTGCGGACATCACTCTT 3') (SEQ ID NO: 2), 500 ng of TMV RNA, and 20 U of *Bst* large fragment. One reaction contained no $MgCl_2$, one contained 1.5 mM $MgCl_2$, and one contained no $MgCl_2$, but 0.5 mM $MnSO_4$ was added. RT was performed at 60°C for 40 minutes.

Five microliters of each of the RT reactions was then amplified by PCR in reactions containing 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 μM (micromolar) each dNTP, 1.5 mM $MgCl_2$, 1.5 Units of *Taq* DNA polymerase, and 12.5 pmoles of both forward and reverse TMV primers having the following sequences: (forward) (5' GCCGGTTTGGTCGTCACGGGC 3') (SEQ ID NO: 3); (reverse) (5' CCCTTTGCGGACATCACTCTT 3') (SEQ ID NO: 4). Thirty-five cycles of amplification were performed with the following cycling profile: denaturation for 1 minute at 92°C, primer annealing for 1 minute at 64°C, and primer extension for 1 minute at 72°C. Ten microliters of each reaction were separated by agarose gel electrophoresis and visualized by ethidium bromide staining and UV transillumination. The 463 bp product was clearly visible in both the reactions performed with $MgCl_2$ and $MnSO_4$ (Figure 3, lanes 2 and 3, respectively). A small amount of product was also produced in the reaction where the reverse transcription was performed without either $MgCl_2$ or $MnSO_4$.

Example 3: Comparison of Mg^{2+} and Mn^{2+} effects on fidelity

Comparisons were made of the rate of misincorporation of nucleotides during RT-PCR amplification. Reactions were performed in the presence of 1.5 mM $MgCl_2$ with or without the addition of 0.5 mM $MnSO_4$, which is required by most thermostable enzymes capable of reverse

transcription. The sequences of resulting products were compared to generate relative mutation rates.

A region of the rabbit tissue factor (RTF) mRNA transcript was used as a template for the misincorporation studies. The RTF-RNA transcript was generated by subcloning an RT-PCR amplified rabbit brain mRNA product into a transcription vector containing a T7 promoter. The forward and reverse RTF primers used for the amplification had the following sequence:
(forward) 5' GGAACCGGTGCAGACACTACAGGTAGAGC 3' (SEQ ID NO: 5) and
(reverse) 5' CCCAAGCTTCAGGCGATGTTCAGG 3' (SEQ ID NO: 6).
Amplification conditions were as follows: reverse transcription was performed at 42°C for 30 minutes, followed by 30 amplification cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds.

The RT-PCR product was ligated into a plasmid by standard methods. The RTF containing subclone was sequenced by standard methods and was verified. T7 RNA polymerase was then used to transcribe from the subcloned RTF plasmid in a standard transcription reaction. The RNA template was quantified by spectrophotometry. Two hundred and fifty picograms of RNA transcript were used in the subsequent RT-PCR amplification reactions.

An 850 nucleotide long region of the rabbit tissue factor (RTF) message was amplified from an RNA template by single step RT-PCR reactions using *Bst* DNA polymerase large fragment for reverse transcription and *Taq* DNA polymerase for subsequent amplification. Reactions contained 50 mM Tris-HCl, (pH 9.0), 20 mM $(NH_4)_2SO_4$, 12.5 mM NaCl, 200 μM (micromolar) each dNTP, 1X MASTERAMP PCR Enhancer (Epicentre Technologies), 12.5 pmoles of both forward and reverse RTF primer having the following sequence:
(forward) 5' CGGCGGCCGCAGACACTACAGGTAGA 3' (SEQ ID NO: 7);

(reverse) 5' GCTCTAGATTCAGGCGATGTTCAGGGGGGA 3' (SEQ ID NO: 8), and 250 pg RTF RNA transcript, and 20 U *Bst* DNA polymerase large fragment and 5 U *Taq* DNA polymerase. One of the reactions also contained 0.5 mM $MnSO_4$. The reagents were incubated for 30 minutes at 60°C to allow reverse transcription, and then PCR was performed using 30 cycles of: 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Products were quantified by fluorimetry and ligated into a pT7.1 plasmid vector. The ligations were transformed into *E.coli* and DNA was purified from 3-5 colonies from each original amplification. The DNA was sequence and compared to the published sequence of RTF.

The sequences were used to determine the relative misincorporation rates of reverse transcription and amplification in the presence of 1.5 mM magnesium +/- 0.5 mM manganese. The number of mutations per base sequenced was .0023 without manganese and was 0.0085 in the presence of manganese. The misincorporation rate was therefore 3.7 fold better when reverse transcription and the subsequent amplification were only dependent on the cation magnesium. Therefore, the presence of manganese adversely affects the fidelity of RT-PCR amplifications.

Example 4: Long RT-PCR

Reverse transcription of a large RNA target was performed to demonstrate the ability of the large fragment of *Bst* DNA polymerase to copy and amplify long regions of RNA when combined with one or more thermostable DNA polymerases.

A 4650 nucleotide long region of tobacco mosaic virus (TMV) RNA was amplified in reactions containing 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM (micromolar) each dNTP, 50 pmoles of TMV2 reverse primer (5' TCGCTTTATTACGTGCCTGC 3') (SEQ ID NO: 9), 200 ng of TMV RNA, 20 U *Bst* DNA

polymerase large fragment, 5 U *Taq* DNA polymerase, and 0.25 U of *Pwo* DNA polymerase. Two reactions were performed, one with and one without 1X MASTERAMP PCR Enhancer (Epicentre Technologies) included in the reaction. RT was performed at 60°C for 30 minutes, followed by 20 cycles of PCR amplification at 92°C for 30 seconds, 62°C for 30 seconds, 72°C for 3 minutes, and then 15 cycles of 92°C for 30 seconds, 62°C for 30 seconds, 72°C for 3.5 minutes plus 15 seconds added per cycle. Ten microliters of each reaction were separated by agarose gel electrophoresis and visualized by transillumination. Some smaller non-specific amplification products are detected, but the expected 4.6 Kb product was produced in the presence of 1X MASTERAMP PCR Enhancer (Epicentre Technologies) (Figure 4, lane 2).

The foregoing examples exemplify various embodiments of the present invention and are not intended to limit the invention, the scope of the invention, and its equivalents being determined solely by the claims.

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